UNIVERSITY OF HELSINKI

Faculty of Biological and Environmental Sciences

The effect of butyrate on the release of serotonin from the gastrointestinal tract

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Helsinki 2019

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Tekijä – Författare – Author Sofie Schubert		
Työn nimi – Arbetets titel – Title The effect of butyrate on the release of serotonin from the gastrointestinal tract		
Oppiaine/Opintosuunta – Läroämne/Studieinriktning – Subject/Study track Neuroscience		
Työn laji – Arbetets art – Level Master´s thesis	Aika – Datum – Month and year May 2019	Sivumäärä – Sidoantal – Number of pages 36

Tiivistelmä–Referat–Abstract

Understanding the link between the gut microbiota, diet and the enteric nervous system is of significant importance in the prevention of gastrointestinal disorders. The aim of the study was to answer two questions: Firstly, is butyrate able to stimulate the luminal release of serotonin? Secondly, in which parts of the gastrointestinal tract does this possibly occur? These questions are of interest, due to the importance of the serotonergic signalling in the enteric nervous system.

We created a luminal perfusion system to investigate the effect of butyrate in the gastrointestinal tract of male Wistar rats (500-550g). We isolated the stomach and 4 cm long segments of the duodenum, jejunum and colon. To our knowledge this form of physiological *ex vivo* studies investigating the entire gastrointestinal tract have not been done previously. The isolated stomach and the isolated intestinal segments were luminally perfused with 100 mM butyrate for 10 min respectively 45 min. The tissues were homogenized after the luminal perfusion. Serotonin and its main metabolite 5-hydroxyindoleacetic acid (5-HIAA) were assayed using commercial ELISA kits.

Our results showed that butyrate significantly stimulates the release of 5-HIAA in the stomach, duodenum, jejunum and colon. Butyrate seems also to have a positive trend-effect on the release of serotonin itself in the stomach, duodenum, jejunum and colon.

Although, there is a future potential for preventing gastrointestinal disorders with the help of diet and gut microbiota, the possible clinical significance of our results should be considered carefully.

Avainsanat – Nyckelord – Keywords
Serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), butyrate, gastrointestinal tract, luminal perfusion system

Ohjaaja tai ohjaajat – Handledare – Supervisor or supervisors
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Säilytyspaikka – Förvaringställe – Where deposited

Muita tietoja – Övriga uppgifter – Additional information

Tiedekunta – Fakultet – Faculty Bio- och miljövetenskapliga fakulteten		Koulutusohjelma – Utbildingsprogram – Degree Programme Utbildningsprogrammet i neurovetenskap
Tekijä – Författare – Author Sofie Schubert		
Työn nimi – Arbetets titel – Title Butyratets inverkan på utsöndringen av serotonin från mag-tarmkanalen		
Oppiaine/Opintosuunta – Läroämne/Studieinriktning – Subject/Study track Neurovetenskap		
Työn laji – Arbetets art – Level Pro gradu	Aika – Datum – Month and year Maj 2019	Sivumäärä – Sidoantal – Number of pages 36

Tiivistelmä–Referat–Abstract

Att förstå kopplingen mellan tarmbakterierna, dieten och det enteriska nervsystemet är mycket centralt vid förebyggande av gastrointestinala sjukdomar. Målet med studien var att ge svar på två frågor: För det första, kan butyrat stimulera den luminala serotoninutsöndringen från mag-tarmkanalen? För det andra, i vilka delar av mag-tarmkanalen inträffar detta i så fall? De här frågorna är av intresse, med tanke på hur viktig serotoninsignaleringen är i det enteriska nervsystemet.

Vi byggde upp ett luminalt perfusionssystem för att undersöka butyratets inverkan i mag-tarmkanalen på Wistar råttor, hane (500-550g). Vi avskilde magsäcken samt 4 cm långa segment av duodenum, jejunum och colon. Så vitt vi vet, har inte denna form av fysiologisk *ex vivo* perfusionsstudie, som undersöker hela mag-tarmkanalen, gjorts tidigare. Den avskilda magsäcken och de avskilda segmenten perfuserades luminalt med 100 mM butyrat under 10 min respektive 45 min. Vävnaderna homogeniserades efter perfusionen. Serotonin samt dess huvudsakliga metabolit, 5-hydroxyindoleacetisksyra (5-HIAA), analyserades med hjälp av kommersiella ELISA kit.

Våra resultat visade att butyrat signifikant stimulerar utsöndringen av 5-HIAA från magsäcken, duodenum, jejunum och colon. Butyrat verkar även ha en positiv trend-effekt på utsöndringen av själva serotoninet från magsäcken, duodenum, jejunum och colon.

Även om det finns en potential för att i framtiden förebygga gastrointestinala sjukdomar med hjälp av diet och tarmbakterier, bör den eventuella kliniska betydelsen av våra resultat tas i betraktande med en viss försiktighet.

Avainsanat – Nyckelord – Keywords
Serotonin (5-HT), 5-hydroxyindoleacetisksyra (5-HIAA), butyrat, mag-tarmkanal, luminalt perfusionssystem

Ohjaaja tai ohjaajat – Handledare – Supervisor or supervisors
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Säilytyspaikka – Förvaringställe – Where deposited

Muita tietoja – Övriga uppgifter – Additional information

Abbreviations

ENS	Enteric nervous system
FFA2	Free fatty acid receptor 2
FFA3	Free fatty acid receptor 3
GPR109A	G-protein coupled receptor 109A
HRP	Horseradish peroxidase
5-HIAA	5-hydroxyindoleacetic acid
5-HIAL	5-hydroxyindole-3-acetaldehyde
5-HT	5-hydroxytryptamine
5-HTOL	5-hydroxytryptophol
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
LDH	Lactate dehydrogenase
Olfr 78	Olfactory receptor 78
SCFA	Short chain fatty acids
TMB	3,3’,5,5’-Tetramethylbenzidine (TMB)

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1. Introduction

The enteric nervous system (ENS) operates by an independent reflex activity and is often described as “the second brain”. By beginning in the oesophagus and extending down to the anus, it governs the function of the entire gastrointestinal tract (1,2). ENS regulates gastrointestinal motility, the peristaltic reflex, blood flow, absorption of nutrients and fluid secretion (2,3). A key neurotransmitter in the ENS is 5-hydroxytryptamine (5-HT), also known as serotonin. 5-HT transfers information from the gastrointestinal tract to the central nervous system and acts as a growth factor, promoting enteric neuronal development and survival (3). The gastrointestinal tract alone stands for approximately 90% of the total body production of 5-HT, while the rest 10% is produced in the central nervous system and in the platelets (4).

Gut microbiota and diet have recently been reported to affect the ENS and the 5-HT signalling in the gastrointestinal tract (3,5-7). Increasing evidence shows that the effect might be through the action of short chain fatty acids (SCFA) (3,6-9). SCFA are the main products in the bacterial fermentation of fibres (10,11) and form a link between the diet, the gut microbiota and the nervous system. This link is of interest, since gut microbiota has received increased attention for its complex influence on the human body and its involvement in several neurological and gastrointestinal diseases (12). Studies have reported changes in the composition of gut microbiota, the ENS and the 5-HT signalling in patients with irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (13-16). Considering the increased incidence of these diseases in the past decade, understanding the link between the gut microbiota, diet and the ENS might be of significant importance in the prevention of gastrointestinal disorders.

1.1. The enteric nervous system (ENS)

The ENS consists of sensory neurons, motor neurons and interneurons organized into two main types of plexuses, the submucosal plexus and the myenteric plexus (Figure 1). The myenteric plexus participates in the control of gastrointestinal motility and is situated between the longitudinal and the circular muscle layers (1,3). The submucosal plexus is located near the muscularis mucosae and can be divided into three separate neuronal sub plexuses: The inner Meissner´s plexus situated directly below the muscularis mucosae, the intermediate plexus situated close to the muscularis mucosae and the outer Henle´s plexus situated directly adjacent to the circular muscle layer of the gastrointestinal tract (2). The main part of the submucosal neurons participate in the control of electrolyte secretion, intestinal epithelia cell proliferation and the modulation of paracellular permeability via control of tight junction associated proteins (2). As in all biological systems there are exceptions, and some of the submucosal neurons extend to the circular muscle layer while some of the myenteric neurons extend to the mucosa (1).

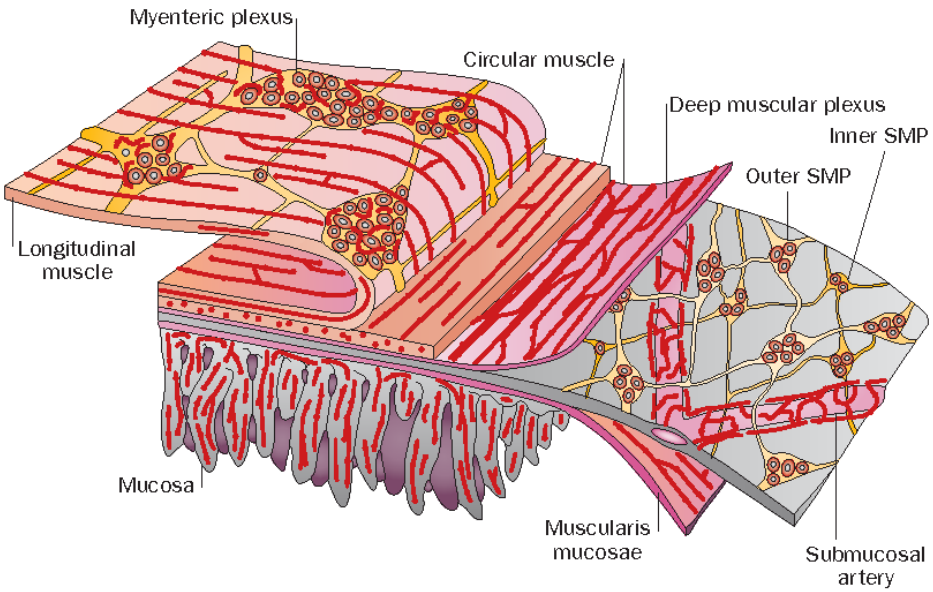

Figure 1. The anatomy of the enteric nervous system. SMP = Submucosal plexus.
Figure from Furness J et al., 2012 (17).

The ENS operates autonomously by an independent reflex activity and responds to mechanical and chemical stimuli in the gastrointestinal tract. Approximately two-thirds of the enteroendocrine cells, specialized hormone and peptide releasing cells found alongside the epithelial cells, synapse with neurons of the ENS (18).

Communication with the central nervous system is, however, important and occurs through the parasympathetic and sympathetic nervous system. Especially the *vagus* nerve plays an important role in the bidirectional communication between the central nervous system and the ENS (12). The *vagus* nerve is the tenth of the cranial nerves and the longest, extending from the brain to the heart, lungs, gastrointestinal tract and other organs in the abdomen. Vagal neurons also seem to synapse with enteroendocrine cells in the gastrointestinal tract, thus possibly forming a direct link between the brainstem and the gastrointestinal tract (18).

1.2. The gut microbiota affects the ENS

The gut microbiota is defined as all bacteria found in the entire gastrointestinal tract. A healthy gastrointestinal tract contains up to 1000 different bacterial species in human and most of the bacteria resides in the colon (12). The gut microbiota is essential for maintaining intestinal homeostasis and health: It participates in the production of vitamin K, vitamin A, biotin, thiamine, folate and riboflavin, takes part in the digestion of fibres and affects the energy balance (12), the immune system (19,20) and the nervous system (3,20-25) (Figure 2).

The gut microbiota affects the ENS via several mechanisms. It modulates the 5-HT signalling in the ENS and stimulates the differentiation as well as the maturation of neurons in the ENS (3). The gut microbiota is also reported to alter gene expression in neurons of the ENS (23) and to stimulate the activity of neurons (21,23). Furthermore, the gut microbiota stimulates the *vagus* nerve (12,18) and inhibits sympathetic neurons originating from the central nervous system (22). The proposed mechanism of action is via the formation of SCFA (21,23).

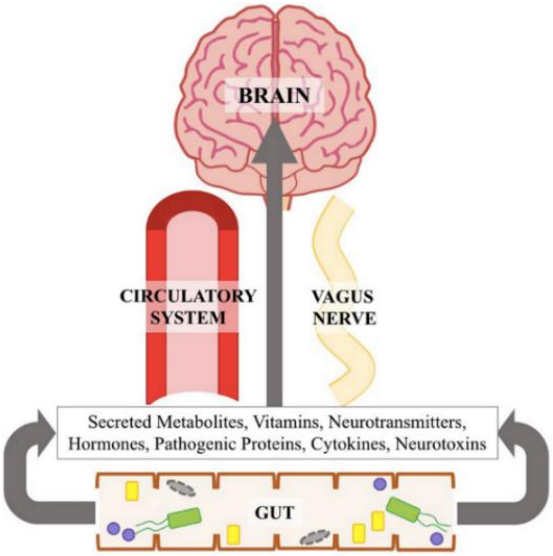

Figure 2. The gut microbiota is important and affects the nervous system via the circulatory system and the *vagus* nerve. Figure from Spielman L et al., 2018 (12).

1.3. SCFA – A link between diet, gut microbiota and the nervous system

SCFA (short chain fatty acids) are defined as a group of molecules consisting of two to six carbon atoms. They are the major products of the bacterial fermentation of large carbohydrate complexes, but also smaller carbohydrate complexes and proteins (10,11). The main SCFA produced in the human colon are acetate, propionate and butyrate in the molar ratio 57:21:22 (10) (Figure 3). The total luminal colonic concentration of SCFA is around 130 mM and the concentration of SCFA in blood is reported to be: portal 375±70 µM, hepatic 148±42 µM and peripheral 79±22 µM (10). It is, however, important to take into account that the concentration of SCFA in the human body varies significantly due to diet and the composition of gut microbiota. Furthermore, the circadian clock appears to affect the intestinal concentration of SCFA, but the mechanisms involved are not known (26).

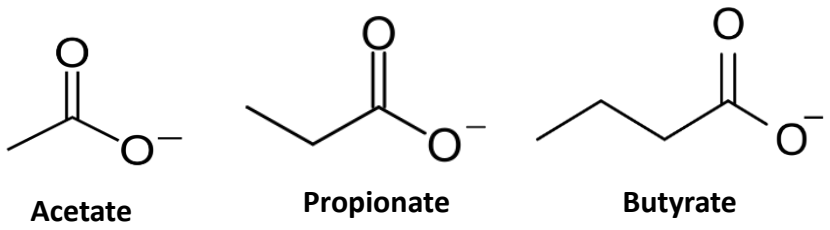

Figure 3. The main short chain fatty acids produced in human: Acetate, propionate and butyrate. They appear as anions in the body, due to the pKa value 5 (27).

SCFA are absorbed from the intestinal lumen and enter the cell in several ways, by passive diffusion through the cell membrane, by active transport via monocarboxylic acid/ketone transporters (28) and by SCFA/HCO3-exchangers (27). SCFA are important energy sources for colonocytes in the gastrointestinal tract but are also able to act as signaling molecules (19,20,25,27) and regulators of gene expression (23,28).

SCFA interact with four transmembrane G-protein coupled receptors: Free fatty acid receptor 2 (FFA2), free fatty acid receptor 3 (FFA3) (19), G-protein coupled receptor 109A (GPR109A) (29) and olfactory receptor 78 (Olfr 78) (30). The receptors FFA2 and FFA3 are the most abundant receptors for SCFA in the gastrointestinal tract and the peripheral nervous system (20,25,27) (Table 1).

Table 1. The presence of receptor FFA3 and FFA2 in the gastrointestinal tract and the peripheral nervous system (20,25,27,31). FFA3 has furthermore been detected on endothelial cells in the vasculature and FFA2 on enteric leukocytes (20). Receptor GPR109A has been detected on epithelial cells in the ileum and colon (29), whereas receptor Olfr 78 has been detected in the renal juxtaglomerular apparatus and the olfactory epithelium (30). Detections were done in the mouse and rat. SG = Sympathetic ganglia.

Region	FFA3	FFA2
Gastrointestinal tract	S cells	-
	L cells	L cells
	Ghrelin cells	-
	G cells	-
	Cholecystokinin cells	-
	Enterochromaffin cells	Enterochromaffin cells
	K cells	K cells
	Substance P cells	-
	D cells	-
	Neurotensin cells	-
Peripheral nervous system	Sensory neurons	-
	Motor neurons	-
	Enteric neurons	-
	Superior cervical ganglion	-
	SG of the thoracic trunk	-
	SG of the lumbar trunk	-
	Prevertebral ganglion	-
	Vagal ganglion	-
	Trigeminal ganglion	-
	Spinal dorsal root ganglion	-

-not detected in the mouse and rat.

1.4. 5-HT in the gastrointestinal tract

5-HT is an important neurotransmitter in the ENS, that was discovered by two independent research investigations (32,33). By activating intrinsic and extrinsic sensory neurons in the ENS, 5-HT participates in the regulation of normal functioning of the gastrointestinal tract, such as peristaltic contractions, intestinal motility and secretion of mucous (4,34). The action of 5-HT is mediated by the 5-HT receptor family 5-HT1-5-HT7 (34). All receptors are transmembrane G-protein coupled metabotropic receptors, except the 5-HT3 receptor, that is a ligand-gated ion channel. The predominant 5-HT receptors in the gastrointestinal tract are 5-HT3 and 5-HT4, located in the myenteric plexus, the submucosal plexus and on the *vagus* nerve (34).

5-HT is synthesized from the essential amino acid tryptophan, that is converted by the rate-limiting enzyme tryptophan hydroxylase to 5-HT (4) (Figure 4). The 5-HT synthesis takes place in the enterochromaffin cells, containing the rate-limiting isoform enzyme tryptophan hydroxylase 1 and in serotonergic neurons of the myenteric plexus containing the rate-limiting isoform enzyme tryptophan hydroxylase 2 (35,36). Most of the 5-HT synthesis takes place in the enterochromaffin cells. Enterochromaffin cells are a type of enteroendocrine cells found alongside the epithelial cells in the gastrointestinal tract (35). 5-HT is released from the enterochromaffin cells by exocytosis from storage granules. The release of 5-HT occurs predominantly from the basal membrane into small blood vessels and the *lamina propria*, where 5-HT can interact with enteric neurones (4,8). Release of 5-HT occurs also from the apical membrane, into the lumen of the gastrointestinal tract (4). The release of 5-HT is triggered by mechanical, neurological and chemical stimuli (4,9).

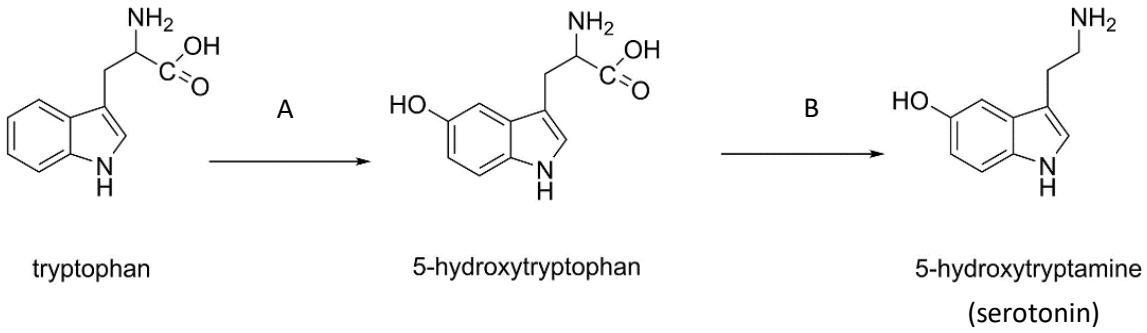

Figure 4. Tryptophan is converted by the rate-limiting enzyme tryptophan hydroxylase (A) to 5-hydroxytryptophan. 5-hydroxytryptohan is further converted to 5-HT by the enzyme L-aromatic amino acid decarboxylase (B). Figure from Szeitz A et al., 2017 (4).

5-HT is metabolized in the gastrointestinal tract, neurons, liver, kidneys and lungs to 5-hydroxy-indole-acetic acid (5-HIAA) and 5-hydroxytryptophol (5-HTOL), which are excreted in the urine (4,37,38) (Figure 5). 5-HIAA is the main metabolite of 5-HT in humans, with urinary concentrations ranging from 5 to 40 μM. The urinary concentration of 5-HTOL is normally low, less than 1% of the 5-HIAA concentration (38).

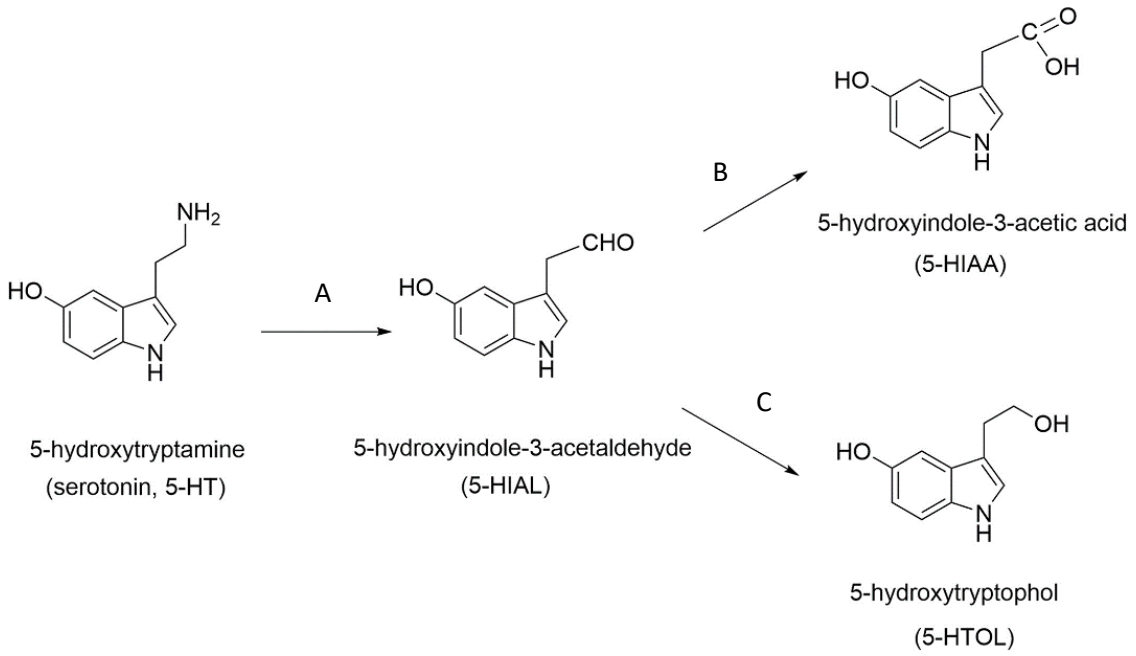

Figure 5. 5-HT is converted by monoamine oxidase A (A) to 5-hydroxyindole-3-acetaldehyde (5-HIAL). 5-HIAL is then further converted to 5-HIAA by aldehyde dehydrogenase (B) or 5-HTOL by alcohol dehydrogenase (C) (37,38). Figure from Szeitz A et al., 2017 (4).

1.5. The gut microbiota affects the synthesis and release of 5-HT

The gut microbiota affects 5-HT signaling in the ENS by inducing *de novo* synthesis of 5-HT and stimulating neuronal and mucosal 5-HT release (3,5-7). The absence of gut microbiota or depletion of gut microbiota with antibiotics decreases colonic tryptophan hydroxylase 1 expression and circulating 5-HT levels (3,6). On the other hand, colonization of germ-free mice with human-derived gut microbiota stimulates colonic motility *in vivo*, increases the colonic tryptophan hydroxylase 1 levels and restores the concentration of 5-HT (3,6,7).

1.5.1. Are SCFA able to stimulate the release of 5-HT and affect the ENS?

Contradictory results exist regarding the effect of SCFA on the release of 5-HT from the gastrointestinal tract. SCFA have been reported to increase 5-HT levels in the duodenum and colon, increase the production of 5-HT in the enterochromaffin cells and to increase the expression of the rate limiting enzyme tryptophan hydroxylase 1 in the 5-HT producing enterochromaffin cells (6-9). SCFA facilitate gene expression by acting as histone deacetylase inhibitors (23,28). Histone deacetylase inhibitors inhibit deacetylation of histone proteins, thereby opening up the chromatin structure and facilitating gene expression. Furthermore, the receptors FFA2 and FFA3 have been detected on 5-HT producing enterochromaffin cells in the mouse duodenum and colon (27,31). Contrary to these findings, SCFA are also reported not to affect the release of 5-HT from isolated primary mouse enterochromaffin cells derived from the duodenum and colon (39).

SCFA both stimulate and inhibit neuronal activity of the ENS. The stimulatory effect of SCFA is believed to occur via the release of 5-HT, since SCFA are reported not to increase motility in tryptophan hydroxylase 1 knock out mice (21). After distribution of SCFA, the release of 5-HT is reported to activate 5-HT3 receptors located on vagal sensory nerve terminals leading to colonic contractions (8) and 5-HT4 receptors located on sensory calcitonin gene-related peptide-containing nerve terminals triggering the peristaltic reflex in the colon (9). In contrast, SCFA have also been reported to inhibit neurons of the ENS by reducing the neuronally mediated contraction of the colon and decrease the peristaltic peak amplitude in the colon (40-43). The inhibitory effects of SCFA are believed to occur via stimulated release of peptide YY, a gastrointestinal hormone inhibiting motility (42) and via activation of FFA3 in the myenteric plexus (44).

1.6. The aim of the study

Most of the studies investigating the effect of SCFA on the release of 5-HT have been performed in the colon. To our knowledge studies investigating this matter along the entire gastrointestinal tract have not been done.

The aim of the study was to answer two questions: Does butyrate stimulate luminal release of serotonin and if so, in which parts of the gastrointestinal tract does this possibly occur? These questions are of interest, due to the conflicting results regarding SCFA:s effect on 5-HT release and the importance of the 5-HT signalling in the ENS. Our choice to study the effect of butyrate is based on the literature, where butyrate has gained the most attention.

We created a luminal perfusion system to investigate the effect of butyrate on 5-HT release from different parts of the gastrointestinal tract in an *ex vivo* setting. Luminal perfusion was previously used successfully in a study investigating the effect of SCFA in the proximal colon (8).

2. Materials and Methods

2.1. Animals

A total of 14 male Wistar rats (500-550g) were obtained from Janvier Labs (Saint-Berthevin, France). The animals were housed in individually ventilated cages in social groups of two rats in a specific-pathogen free animal facility with a 12-h dark/light cycle, an average room temperature of +22°C, and a relative humidity of 55%. Rat chow (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories, Madison, WI, USA) and tap water were available *ad libitum.*

Before euthanasia, the animals were fasted for 4 h (6 am -10 am) with free access to water to minimize the amount of chymus in the stomach and intestines. The animals were euthanized by decapitation with a guillotine. The abdomen was opened and the stomach, duodenum (directly after the stomach), jejunum (10 cm from duodenum) and colon (3 cm from the caecum) were isolated. The isolated segments of duodenum, jejunum and colon were 4 cm long. To minimize tissue damage, both the animals and intestinal segments were kept on ice during tissue harvesting. The isolated intestinal segments were kept in small dishes containing Krebs buffer (116.0 mM NaCl, 4.6 mM KCl, 2.6 mM $CaCl_2 \cdot 2\ H_2O$, 1.2 mM $MgSO_4 \cdot\ 7\ H_2O$, 24.9 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 2.5 mM glucose, pH 7.5, +37°C, saturated with 95% O_2 and 5% CO_2 gas). The isolated stomach and the intestinal segments of duodenum, jejunum and colon were cleaned from chymus by careful injections of Krebs buffer using a syringe. The isolated stomach and the intestinal segments were weighted and stored on individual Petri dishes in cold Krebs buffer at +4°C before mounting them into the luminal perfusion system.

All procedures were carried out in accordance with the ethical approval ESAVI 10698/04.10.07/2017.

2.2. Luminal perfusion of the stomach and the isolated segments of duodenum, jejunum and colon

2.2.1. Setting up the luminal perfusion system

The stomach and isolated segments of duodenum, jejunum and colon were used *ex vivo* in a luminal perfusion system created by Sofie Schubert and laboratory technician Päivi Leinikka. The perfusion system consisted of a thermostatically controlled water bath (Grant Instruments, Royston, England), four carbogen tubes (95% O_2 and 5% CO_2 gas), two perfusion fluids (Krebs buffer only and sodium salt of butyric acid [Sigma-Aldrich, St. Louis, MO, USA] dissolved in Krebs buffer), perfusion tubes (ID 1.85 mm and ID 2.3 mm), a peristaltic pump (Ismatec, Barrington, IL, USA), thin thread (DG, Wayne, NJ, USA), a heating block and glass tubes (Figure 6). The thermostatically controlled water bath was used to prewarm the perfusion fluids to +37°C. The carbogen tubes were used to ensure a sufficient amount of oxygen in the perfusion fluids. The peristaltic pump was linked to four perfusion tubes, one for each sample. The perfusion flow rate was 1 ml/min. The smaller perfusion tubes (ID 1.85 mm) were used to transport perfusion fluid into the intestinal segments, while the wider perfusion tubes (ID 2.3 mm) were used to transport the perfusion fluid out of the intestinal segments. Thin tread was used to fasten the intestinal segments to the perfusion tubes. The heating block was used to keep the intestinal segments at +37°C during the experiment. On the heating block was a dish for each sample, containing 0.9% NaCl solution. The 0.9% NaCl solution was used to maintain the osmotic pressure in the samples. The glass tubes were used to collect the luminal perfusion samples.

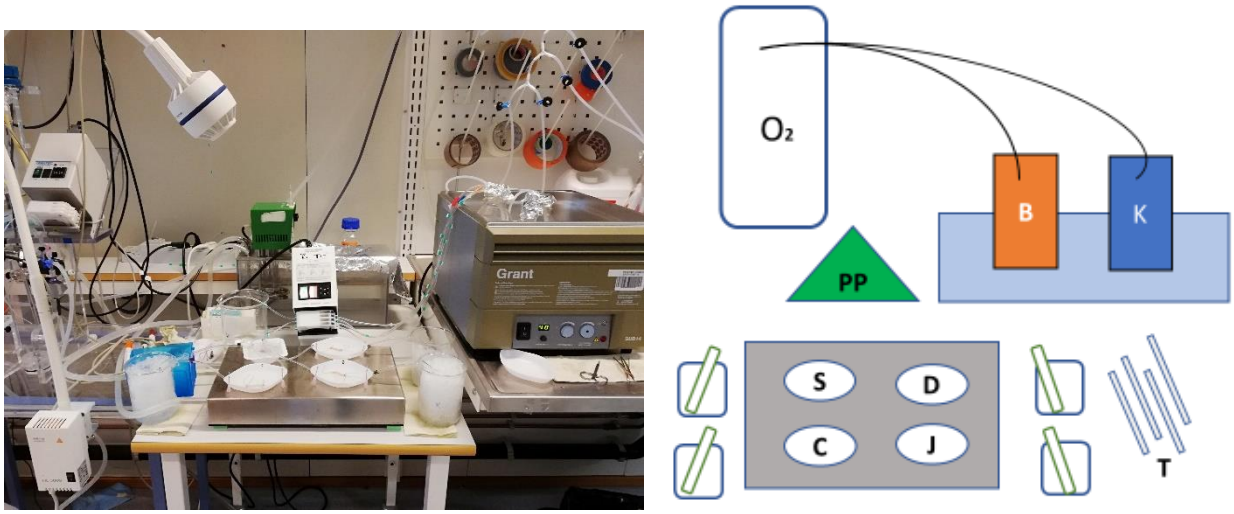

Figure 6. The luminal perfusion system created by Schubert and Leinikka. O2 = 95% O_2 and 5% CO_2 gas, B = butyrate 100 mM, K = Krebs buffer, PP = peristaltic pump, T = perfusion tubes, S = stomach, D = isolated segment of duodenum, C = isolated segment of colon, J = isolated segment of jejunum.

2.2.2. Testing the luminal perfusion system: Pilot experiments on butyrate concentration and time dependency

The perfusion fluids used were oxygenated Krebs buffer (+37°C, pH 7.5) and oxygenated Krebs buffer containing three different concentrations (1 mM, 10 mM and 100 mM) of sodium butyrate (+37°C, pH 7.5). The perfusion flow rate was maintained at 1 ml/min.

The isolated intestinal segments were mounted into the luminal perfusion system in the following order: Stomach, duodenum, colon and jejunum. Thin thread was used to fasten the intestinal segments to the perfusion tubes in the physiological direction. The flow rate was checked separately for each intestinal segment before beginning of the sample collection from the perfusion system. The samples from the perfusion system were collected during a time period of 60 min at 5 min intervals (Table 2). The collected perfusion samples were kept in ice and centrifuged with Multifuge 3L-R (Heraeus, Osterode, Germany) for 15 min at 20000 rpm at +4°C. The supernatant was collected, and pH was measured for each sample, as well as for the perfusion fluids, with the use of pH paper (pH-Fix 7.0-14, Fisherbrand). The supernatant was then stored in -80°C. The tissue samples were carefully taken from the luminal perfusion system, weighted and stored in -80°C.

Table 2. The time points used in the pilot experiment.

Time points & perfusion fluid	Aim
0-5 min, Krebs buffer	Wash the tissue
5-10 min, Krebs buffer	Wash the tissue
10-15 min, Krebs buffer	Used as baseline
15-20 min, 1 mM butyrate / Krebs buffer for controls	Ensure that 1 mM butyrate reaches the intestinal segment in time for the next sample
20-25 min, 1 mM butyrate / Krebs buffer for controls	Study the effect of 1 mM butyrate
25-30 min, 10 mM butyrate / Krebs buffer for controls	Ensure that 10 mM butyrate reaches the intestinal segment in time for the next sample
30-35 min, 10 mM butyrate / Krebs buffer for controls	Study the effect of 10 mM butyrate

35-40 min, 100 mM butyrate / Krebs buffer for controls	Ensure that 100 mM butyrate reaches the intestinal segment in time for the next sample
40-45 min, 100 mM butyrate / Krebs buffer for controls	Study the effect of 100 mM butyrate
45-50 min, Krebs buffer	Used as an intermediate sample, butyrate still in the tissue
50-55 min, Krebs buffer	Study changes in the release of 5-HT after perfusion with butyrate
55-60 min, Krebs buffer	Study changes in the release of 5-HT after perfusion with butyrate

If clogging and swelling of the isolated stomach or the isolated segments of the duodenum, jejunum and colon occurred during the perfusion experiment, the angles of the perfusion tubes were corrected to more optimal and a syringe was used to remove the blockade.

2.2.3. The acute and prolonged effects of butyrate on the 5-HT release

The perfusion fluids used were the same as in the pilot experiments, Krebs buffer and a 100 mM solution of butyrate. Also, the perfusion flow rate was the same as in the pilot experiments (1 ml/min).

The experiments were done in the same way as the pilot experiment, with a few exceptions: The isolated stomach was first mounted into the perfusion system, while the isolated segments of duodenum, jejunum and colon were kept in cold oxygenated buffer in the refrigerator. The intestinal segments where then mounted one by one into the perfusion system. The collection of samples from the luminal perfusion system were done at 10 min intervals (Table 3). The samples were collected in glass tubes containing 0.1% ascorbic acid and the tubes were covered with aluminium foil to prevent oxidation of 5-HT. pH was not measured.

Table 3. The time points used for studying the acute effect of butyrate (I) and the prolonged effect of butyrate (II) on the 5-HT release.

Time points & perfusion fluid	Aim I	Aim II
0-5min, Krebs buffer	Wash the tissue	Wash the tissue
5-15 min, Krebs buffer	Used as baseline	Used as baseline
15-20 min, butyrate / Krebs buffer for controls	Ensure that butyrate reaches the tissue in time for the next sample	Ensure that butyrate reaches the tissue in time for the next sample
20-30 min, butyrate / Krebs buffer for controls	Study the acute effect of butyrate	Study the effect of butyrate
30-40 min, I. Krebs buffer II. butyrate / Krebs buffer for controls	Used as an intermediate sample, butyrate still in the tissue	Study the effect of butyrate
40-50 min, I. Krebs buffer II. butyrate / Krebs buffer for controls	Used as a carry-over sample, butyrate not in the tissue	Study the prolonged effect of butyrate
50-60 min, I. Krebs buffer II. butyrate / Krebs buffer for controls	Study changes in the release of 5-HT after perfusion with butyrate	Study the prolonged effect of butyrate

2.3. The oxidation rate of 5-HT

Two identical collecting glass tubes containing 5-HT diluted in oxygenated Krebs buffer (+37°C, pH 7.5) were used for the experiment. One of the tubes were covered with aluminium foil, the other one was not covered with aluminium foil. A baseline sample was taken from each collecting tube before beginning of the experiment. Samples were then taken every 15 min. The total time of the experiment was 60 min to resemble the collection of samples from the luminal perfusion system. Likewise, the tubes were stored in ice during the entire experiment, as in the perfusion experiment. The samples were stored in -80°C for the 5-HT assay.

2.4. Homogenisation of the stomach and the isolated segments of duodenum, jejunum and colon

A 0.5 cm piece was taken from the middle of the isolated stomach and the isolated segments of duodenum, jejunum and colon. The pieces where put in 500 µl ELISA buffer enriched with ascorbic acid (32 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.46 mM KH_2PO_4, 0.001% Tween, 0.1% ascorbic acid, pH 6.3). Ascorbic acid was used to prevent 5-HT from oxidation. The samples were homogenized with Precellys 24 (Bertin Technologies, Montigny le Bretonneux, France) for 3 x 20 sec, 5500 rpm. To ensure that the cells were broken, the samples were sonicated for 10 sec, 20% with a VC 505 sonicator (Sonics and materials, Newtown, CT, USA). The sonicated samples were centrifuged for 15 min at 11000 rpm at +6°C with Centrifuge 5415D (Eppendorf, Hamburg, Germany). The supernatant was collected and stored in -80°C for the 5-HT assay. The samples were kept in ice during the whole procedure.

2.5. Measurement of 5-HT

The 5-HT content of the collected samples from the luminal perfusion experiments and the 5-HT content of the supernatant from the homogenisation experiment were assayed with a 5-HT high-sensitive ELISA kit (DLD, Hamburg, Germany). From the luminal perfusion experiments, five sample-collection time points were chosen: 5-15 min, 20-30 min, 30-40 min, 40-50 min and 50-60 min. The sample 5-15 min was used as a baseline. The collected samples from the luminal perfusion experiments were diluted with assay buffer 1:2 (duodenum, jejunum and colon) and 1:5 (stomach). The supernatant from the homogenisation experiment were diluted 1:100 and 1:200 (stomach, duodenum, jejunum and colon). The 5-HT content in the samples underwent an acetylation reaction, were 5-HT was chemically derivatized to N-acetyl-5-HT. N-acetyl-5-HT competed for a fixed number of antiserum binding sites until the system was in equilibrium. Free antigen and antiserum complexes were removed by washing. Goat anti-rabbit-IgG-peroxidase was used to detect the N-acetyl-5-HT antibody, bound to the solid phase N-acetyl-5-H. 3,3',5,5'-Tetramethylbenzidine (TMB) was used as substrate, and the TMB / peroxidase reaction was monitored at 450 nm.

2.6. Measurement of 5-hydroxyindoleacetic acid (5-HIAA)

The 5-HIAA content of the collected samples from the luminal perfusion experiment and the supernatant from the homogenisation experiment were assayed with a 5-HIAA ELISA kit (Nordic BioSite AB, Täby, Sweden). Only samples from the luminal perfusion experiment, where the prolonged effect of butyrate had been studied, were used. The same sample-collection time points as in the 5-HT assay, were used from the luminal perfusion experiment. 5-HIAA in the samples competed with a fixed amount of 5-HIAA on the solid phase for sites on the biotinylated detection antibody. Unbound sample and excess conjugate were washed away. Horseradish peroxidase (HRP)-Streptavidin was added, where after the reaction plate was incubated. TMB was used as substrate and was added after the incubation. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution. The colour change was monitored at 450 nm.

2.7. Measurement of lactate dehydrogenase (LDH)

The LDH content of the collected samples from the luminal perfusion pilot experiments was assayed by a LDH kit (Sigma-Aldrich, St. Louis, MO, USA). The collected samples from the luminal perfusion experiments were diluted with assay buffer 1:5 (stomach, duodenum, jejunum and colon). LDH reduced NAD to NADH, which was monitored at 450nm.

2.8. Statistical analysis

Normality of the data sets was tested with the Kolmogorov-Smirnov test. Based on these analyses the data sets from the luminal perfusion experiments were analysed using two-way ANOVA with Bonferroni post-hoc test and the data sets from the homogenisation experiment were analysed using independent samples *t*-test. The data are expressed as means. The degrees of freedom (Df) are reported in the two-way ANOVA analyses. Statistical calculations and figures of the results were made by GraphPad Prism 5 (GraphPad Software Incorporated, La Jolla, CA, USA). Data were considered statistically significant when $p < 0.05$.

3. Results

3.1. Pilot experiments on butyrate concentration and time dependency

3.1.1. Setting up the luminal perfusion system

The pilot experiments showed that the most optimal concentration of butyrate for the luminal perfusion experiment is 100 mM. The 100 mM butyrate solution had the strongest effect on the release of 5-HT compared to 1 mM and 10 mM solutions. The 5 min period for sample collection turned out to be too short and showed considerable variation in the concentration of 5-HT. Thus, it was more profitable to collect samples in periods of 10 min.

The isolated stomach took the longest time to mount into the perfusion system, due to the very thin oesophagus and the contracting pylorus. On the other hand, when mounted into the perfusion system, the isolated segments of duodenum and colon were more susceptible to tissue damage than the stomach and jejunum. Thus, we opted to mount the stomach first into the perfusion system, while keeping the other intestinal segments at + 4 ºC to minimize the risk for tissue damage.

No changes in the pH value 7.5 of the collected perfusion samples nor of the perfusion fluids were observed during the experiment, even after administration of butyrate. This result indicates that it was not necessary to measure the pH value for each collected perfusion sample.

3.1.2. 5-HT is susceptible to oxidation

A distinct reduction in the level of 5-HT was seen after 45 min, without any added compounds (Figure 7). This is of importance to notice, because the collection of samples from the luminal perfusion system takes 60 min and the total length of the luminal perfusion experiment is 120 min. The results showed that 5-HT is susceptible to oxidation and therefore, the sample collecting tubes should be covered with aluminium foil. To further minimize the risk of 5-HT oxidation, an antioxidant (ascorbic acid 0.1%) should be added to the collecting tubes already in the beginning of the experiment. The tubes should also be kept in ice during the entire experiment.

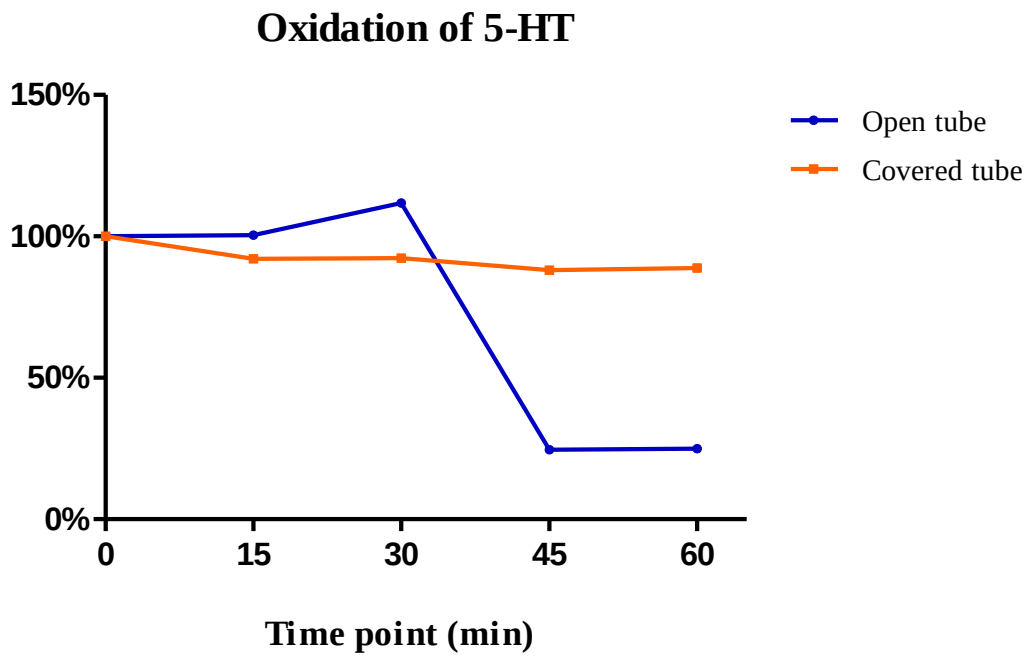

Figure 7. 5-HT is susceptible to oxidation and a reduction in the amount of 5-HT was observed after 45 min. Covered tube = tube covered with aluminium foil, open tube = tube without aluminium foil.

3.1.3. Viability of the isolated stomach and the isolated segments of duodenum, jejunum and colon

The concentration of lactate dehydrogenase (LDH) in the collected luminal perfusion samples was measured to verify the viability of the isolated intestinal segments during the luminal perfusion experiment. LDH is an oxidoreductase enzyme, released when tissue damage occurs. The LDH concentrations did not show significant changes during the experiment, nor did they correlate with the released amount of 5-HT (data not shown). Thus, it was used only in the pilot experiments as an indicator of viability, but not in the experiments investigating the acute and prolonged effect of butyrate.

3.2. The acute effect of butyrate

Luminal perfusion of the isolated stomach and the isolated segments of duodenum, jejunum and colon with 100 mM butyrate for 10 min did not increase the release of 5-HT statistically compared to perfusion with Krebs buffer only (Figure 8).

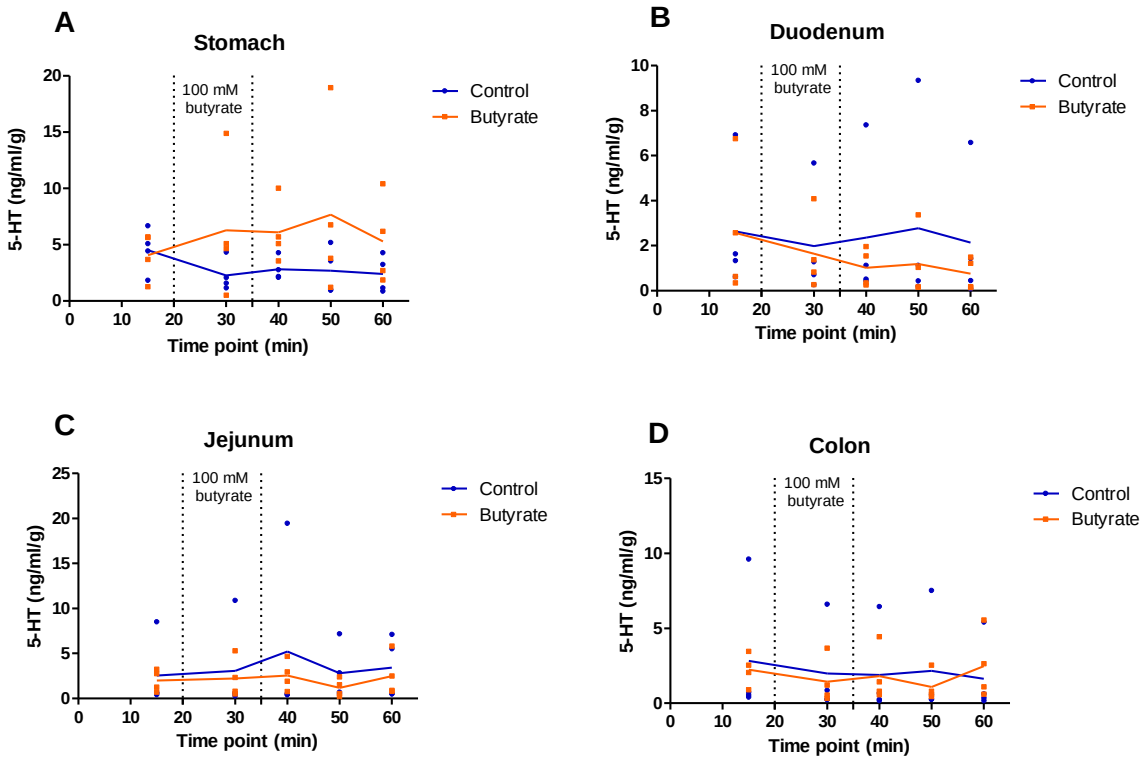

Figure 8. Luminal perfusion with butyrate for 10 min in the stomach (A), duodenum (B), jejunum (C) and colon (D). A-D. Butyrate seems to have a positive trend-effect on the release of 5-HT in the stomach, where the release of 5-HT is the highest. A similar trend-effect cannot be seen in the duodenum, jejunum and colon (n = 8, Df =24, $p>0.05$, two-way ANOVA).

3.3 The prolonged effect of butyrate

3.3.1 Butyrate has a positive prolonged trend-effect on the release of 5-HT in the gastrointestinal tract

Luminal perfusion with butyrate for 45 min did neither increase the release of 5-HT in the isolated stomach nor in the isolated segments of duodenum, jejunum and colon (Figure 9).

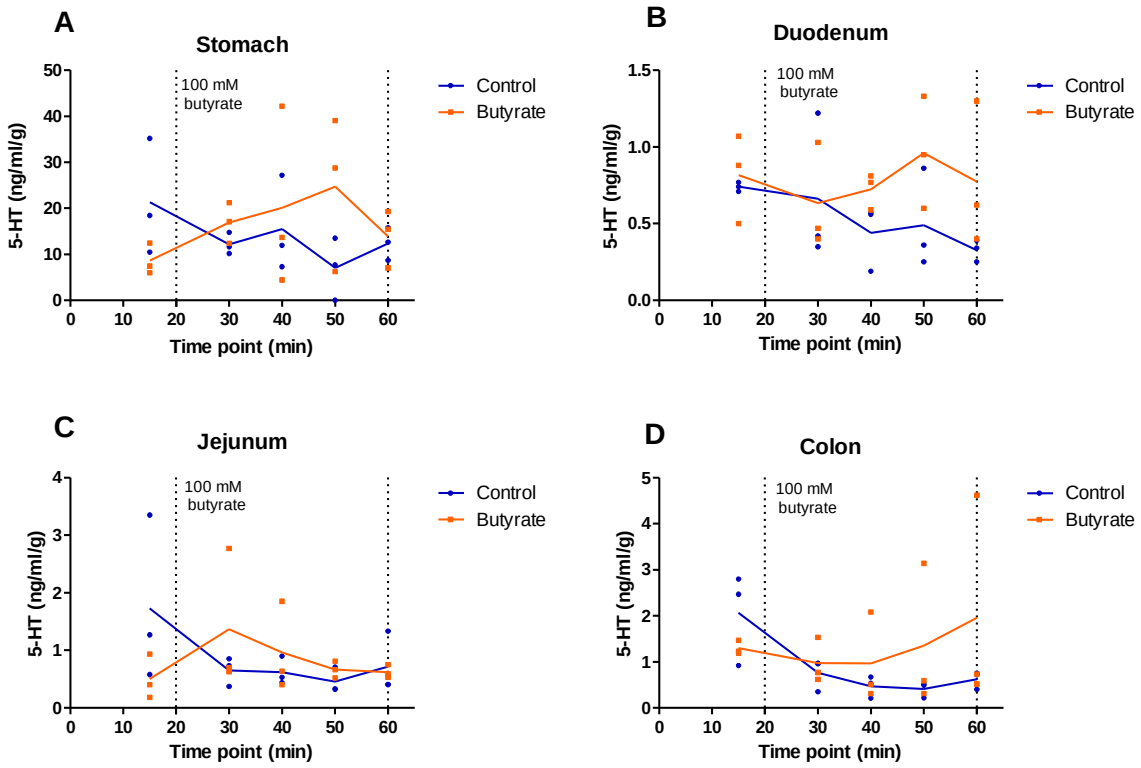

Figure 9. Luminal perfusion with butyrate for 45 min in the stomach (A), duodenum (B), jejunum (C) and colon (D). A-D. Butyrate did not significantly increase the release of 5-HT. A positive trend-effect of butyrate on the 5-HT release can be seen (n = 6, Df =16, $p>0.05$, two-way ANOVA).

3.3.2. 5-HT is released out of the tissue from the isolated gastrointestinal segments

The homogenisation experiment was done to determine whether 5-HT is released into or out of the tissue of the isolated stomach and the isolated segments of duodenum, jejunum and colon. Butyrate did not increase the tissue concentration of 5-HT in the stomach, duodenum, jejunum or colon (Figure 10), indicating that 5-HT is released into the intestinal lumen.

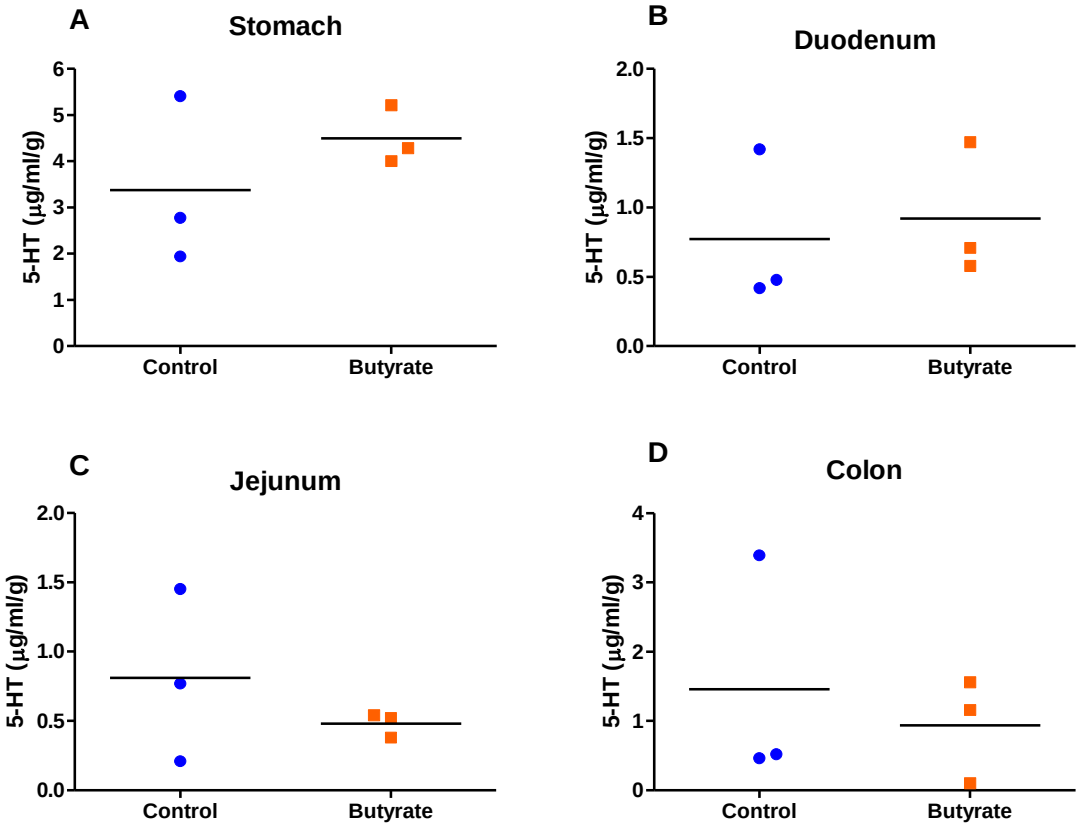

Figure 10. The concentration of 5-HT in the homogenised stomach (A), duodenum (B), jejunum (C) and colon (D) after perfusion with butyrate for 45 min. A-D. Butyrate did not significantly increase the tissue concentration of 5-HT (n=6, p>0.05, independent samples *t*-test).

3.3.3. Butyrate stimulates significantly the release of 5-HIAA from the gastrointestinal tract

5-HT is metabolised to stable 5-HIAA, thus the release of 5-HIAA was analysed from the luminal perfusate as an indicator of 5-HT release. Butyrate stimulated significantly the release of 5-HIAA from the isolated stomach and isolated segments of duodenum, jejunum and colon (Figure 11).

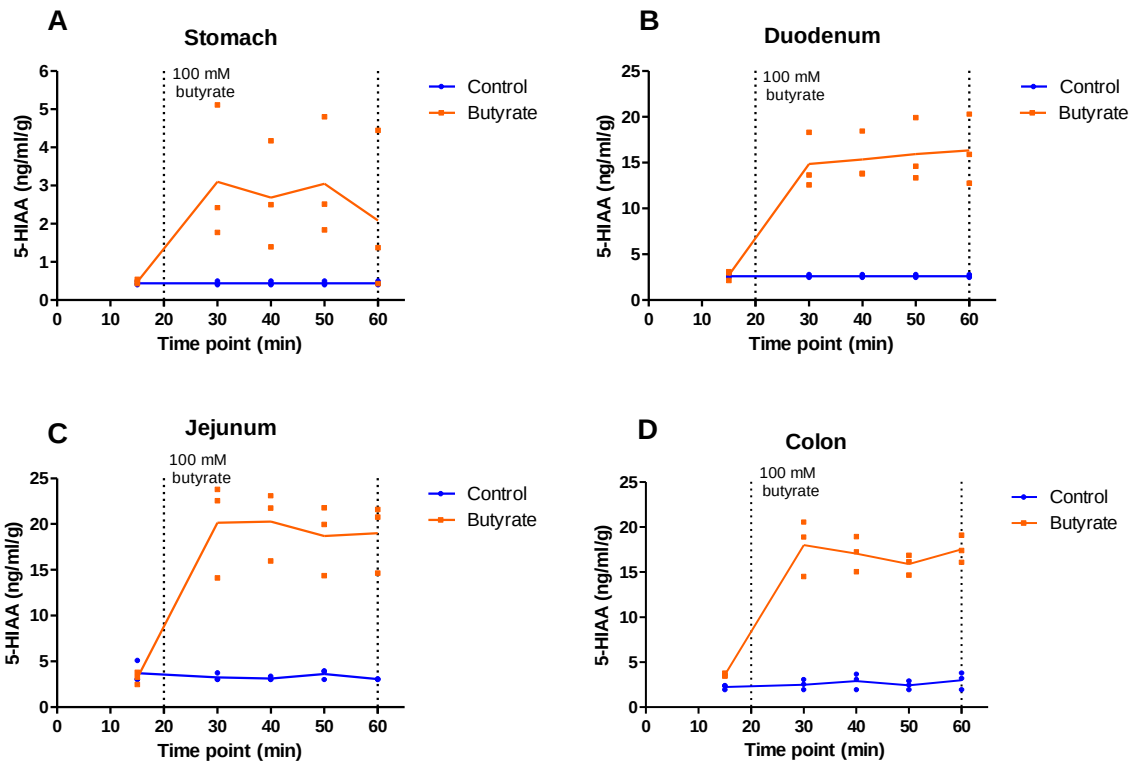

Figure 11. The release of 5-HIAA in the stomach (A), duodenum (B), jejunum (C) and colon (D) after perfusion with 100 mM butyrate for 45 min. A. The concentration of 5-HIAA was significantly increased by butyrate in the stomach (p=0.048). The concentration of 5-HIAA was the lowest in the stomach, compared to the duodenum, jejunum and colon. B-C. A significant increase in the concentration of 5-HIAA was observed in all isolated intestinal segments, duodenum: p=0.0024, jejunum: p=0.0030 and colon: p=0.0001, (n=6, Df=16, two-way ANOVA).

4. Discussion

4.1. General discussion

The aim of the study was to answer two questions: Firstly, is butyrate able to stimulate the luminal release of 5-HT? Secondly, in which parts of the gastrointestinal tract does this possibly occur? These questions are of interest, due to the importance of the 5-HT signalling in the enteric nervous system. Our results show that butyrate significantly stimulates the release of 5-HIAA, the main metabolite of 5-HT, in the stomach, duodenum, jejunum and colon. Butyrate seems also to have a positive trend-effect on the release of 5-HT itself in the stomach, duodenum, jejunum and colon, although, no significant findings were given.

There are conflicting results regarding SCFA effect on the 5-HT release from the gastrointestinal tract. Fukumoto et al. (2003) reported a stimulatory effect in the rat proximal colon *ex vivo*, when administrating 50-200 mM SCFA for 9 min with luminal perfusion (8). They used a mixture of acetate, propionate and butyrate in the molar ratio of 65:20:15. Grider et al. (2007) reported likewise a stimulatory effect of SCFA on the release of 5-HT (9). They used a three-compartment flat-sheet preparation of the distal rat colon. Concentrations of 5 mM acetate, propionate and butyrate were separately administrated for 15 min to the preparation. Contradictory, Martin et al. (2017) reported no effect of SCFA on the release of 5-HT from isolated primary mouse enterochromaffin cells derived from the duodenum and colon (39). Martin et al. used a 2 h incubation and administered separately 1-100 mM acetate, 1-30 mM propionate and 1-30 mM butyrate to the isolated enterochromaffin cells. The difference in the reported results is likely due to the use of different methods, different tissue preparations and different concentrations of SCFA.

Our results support the theory of SCFA stimulating the release of 5-HT (6-9). In line with Fukumoto et al. we created a luminal perfusion system and used *ex vivo* luminal perfusion as the main method (8). In contrast to Fukumoto et al. and Grinder et al. we perfused the isolated stomach and the isolated segments of duodenum, jejunum and colon for 10 min and 45 min with 100 mM butyrate. The longer perfusion time and perfusion with a high concentration of butyrate were motivated after conducting the pilot experiments. The 100 mM butyrate solution had the strongest effect on the release of 5-HT compared to 1 mM and 10 mM solutions in the pilot experiments.

In line with previous studies we showed that butyrate significantly stimulates the release of 5-HIAA, the main metabolite of 5-HT, in the stomach, duodenum, jejunum and colon. Butyrate seems also to have a positive trend-effect on the release of 5-HT itself. We were not able to detect any effect of butyrate in the tissue concentration of 5-HT in the stomach, duodenum, jejunum or colon, indicating that 5-HT might be released into the intestinal lumen. The release of 5-HT is reported to occur predominantly from the basal membrane into small blood vessels and the *lamina propria* (4). Therefore, we were expecting a greater effect of butyrate in the tissue concentration of 5-HT. One reason why we were not able to detect any effect of butyrate might be due to the *ex vivo* preparation. No vasculature was present in the isolated intestinal segments, which might hinder 5-HT from reaching the deeper layers of the tissues.

4.2. Limitations

The luminal perfusion system we created possessed certain limitations that should be considered when interpreting our results. Parts of the mucosa could have been damaged in the stomach and the isolated segments of duodenum, jejunum and colon during the preparation and luminal perfusion. This is problematic, since the 5-HT producing enterochromaffin cells are located in the mucosa. We measured the concentration of LDH in the collected luminal perfusion samples to verify the viability of the isolated intestinal segments, but we did not make any microscopical evaluations after preparation and luminal perfusion.

The stomach and isolated segments of duodenum, jejunum and colon were at risk of swelling due to the luminal perfusion. This might affect the results, since the release of 5-HT is triggered by mechanical stimuli (4,9). Likewise, the Krebs buffer used as perfusion fluid contained calcium and glucose, both of which have been reported to stimulate the release of 5-HT from enterochromaffin cells *in vitro* (35,39). The basal level of 5-HT might therefore be elevated.

We suspect some level of cross-reaction between butyrate and the 5-HIAA ELISA kit, as the results of the 5-HIAA analysis showed exceptionally low variation. Despite our best efforts, we could not verify this matter at this time, but it should be investigated further in the future.

In contrast to the results of the 5-HIAA analysis, there was a high variation in the 5-HT release between individual animals. The high variation might explain why we only saw a positive trend-

effect in the release of 5-HT when investigating the acute and prolonged effects of butyrate but were not able to reach statistical significance.

The circadian clock is reported to affect the concentration of SCFA and the expression of receptor FFA3 in the colonic myenteric plexus in mice (26). This interesting finding leads to the question whether the expression of FFA3/2 on the enterochromaffin cells is controlled by the circadian clock in rats. If so, that may affect our results since rats are nocturnal animals and our animals were euthanised at 10 am. The expression of the receptors for SCFA are presumably low in the morning, which would lead to less binding of SCFA to the receptors on the enterochromaffin cells and thereby to decreased release of 5-HT. If the hypothesis is confirmed, this might also explain why we saw only a positive trend-effect in the 5-HT results.

4.3. Clinical aspects

In IBD and IBS patients, changes in the composition of gut microbiota, the enteric nervous system and the 5-HT signalling have been reported (13-16). The most marked changes in the 5-HT signalling are altered 5-HT synthesis (13,14) and reduced tissue level of 5-HT (16). Our findings indicate that butyrate increases the release of 5-HT in the gastrointestinal tract and is thereby possibly able to increase gut motility. Particularly, a stimulated release of 5-HT would be helpful in increasing the colonic transit in IBS patients suffering from constipation and patients with chronic constipation (14). However, an increased 5-HT level could also be detrimental. In experimental colitis, 5-HT is reported to increase the production of proinflammatory cytokines from macrophages in mice (15) and to decrease bone formation in mice (127). One should, however, consider that results from animal experiments cannot directly be implemented on humans.

5. Conclusion

Understanding the link between the gut microbiota, diet and the enteric nervous system might be of significant importance in the prevention and treatment of gastrointestinal disorders. This study showed that butyrate increases the release of 5-HIAA, the main metabolite of 5-HT, from the stomach, duodenum, jejunum and colon. An important benefit of our study was to create a luminal perfusion system for the *ex vivo* investigations.

Butyrate seems also to have a positive trend-effect on the release of 5-HT itself in the stomach, duodenum, jejunum and colon. If this trend-effect could be confirmed by *e.g. in vivo* animal experiments, we should apply these observations in humans with gastrointestinal distress. Although, there is a future potential for preventing gastrointestinal disorders with the help of diet and gut microbiota, the clinical importance of our results should be considered carefully.

6. Grants

This study was kindly supported by Einar och Karin Stroems stiftelse; Ellen, Hjalmar och Saga Waselius stipendiefond and Nylands Nation.

7. Acknowledgements

My sincere thanks to my supervisors Dr. Richard Forsgård, Prof. Riitta Korpela and Prof. emeritus Heikki Vapaatalo. Thank you, Riku, for your good ideas and for always helping me out. Thank you, Riitta and Heikki, for our inspiring meetings and all your valuable comments.

I would like to thank Päivi Leinikka and Hanna Launonen for teaching and helping me in the laboratory. Thank you, Päivi, for sharing your broad knowledge and vast experience in finding solutions in everyday problems in the laboratory and for our nice morning discussions. Thank you, Hanna, for our many laughs together.

My warmest thanks to my family and friends for all your support and for always being there for me.

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